DEMONSTRATION PROTOCOL

Bioremediation

This protocol is a guide to certifying bioremediation technologies. The protocol delineates information needs, minimum criteria requirements, and a process to be followed in demonstration microbial bioremediation processes.

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CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY

ACCEPTANCE CRITERIA, PERFORMANCE AND PROCESS GUIDANCE FOR THE CERTIFICATION OF BIOREMEDIATION TECHNOLOGIES

Prepared by

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for
California Environmental Protection Agency
California Environmental Technology Certification Program

INTRODUCTION

Program Background

In 1994, the California Environmental Protection Agency's Department of Toxic Substances Control was granted authority to certify the performance of hazardous waste-related technologies, including technologies for pollution prevention, hazardous waste treatment (except incineration), and measurements and monitoring related to hazardous wastes. Authorizing legislation was California Assembly Bill 2060 (Weggeland 1993), now Section 25200.1.5 of the California Health and Safety Code. It is the purpose of the new program to facilitate the acceptance by users and regulatory agencies of novel environmental technologies that are effective, reliable, and safe. It is the purpose of the certification program to eliminate redundant field validations and to simplify regulatory permitting. The authorizing statute specifically states that technologies be evaluated for efficacy and reliability, and that they must not pose a significant potential hazard. The program investigates the soundness of the scientific and engineering basis of technologies proposed for certification. The program's working definition of "technology" is *"a system consisting of equipment and/or materials, knowledge related to its application, a quality assurance system on the part of the manufacturer, and quality assurance practiced on the part of the user."* The certification program is funded through fees from technology proponents.

A key requirement for certification is validation of a technology either by the State or by a qualified independent third party. Besides in-house experts, the program has access to U.S. Government laboratories, the University of California, reference laboratory services, and demonstration sites.

The California hazardous waste technology certification program has the status of pilot program in joint technology verifications with the U.S. Environmental Protection Agency and the U.S. Department of Energy, and other U.S. and state jurisdictions. The program works towards recognition across state and international boundaries. It recognizes the value of international voluntary standards of quality (ISO-9000) and the potential role of technology certification in environmental management under the ISO-14000 series of standards.

The Cal/EPA certification process consists of an eligibility review on the basis of information received from the technology proponent and an analysis of available information on the technology's scientific and engineering principles. Existing validation data, including those obtained by the proponent, are reviewed as the basis for the development of performance claims by the proponent. For these performance claims, independent, quality-controlled verification is sought, typically through field testing. The results are evaluated and described in an Evaluation Report. After successful verification of the proponent's claims the Department prepares and publishes a certification statement which sets out the Department's findings on efficacy, reliability, and protectiveness of the technology, and the prospective, recommended range of application, including limitations. For each technology accepted into the program, the verification process is individually tailored to produce the remaining necessary information.

Since 1995, the California Air Resources Board received authorization for a program for

the precertification of air pollution control technologies. Since 1996, new legislation calls for a coordinated multi-media environmental technology certification program, which will involve all boards and agencies of the California Environmental Protection Agency.

The Case for Bioremediation

Bioremediation and bioremediation-related technologies are among the most promising and cost-effective of the hazardous waste treatment technologies. They allow the destruction of many organic contaminants in an environmentally benign way, and the concentration in a smaller volume, and fixation in less soluble form, of many toxic metals. Yet until now, the evaluation of bioremediation technologies has been difficult for lack of thoroughly documented field experiments, lack of a rigorous, multidisciplinary approach, and the lack of powerful diagnostic tools. With changes on all these fronts there is heightened confidence that evaluation ("validation," "verification," "authentication") of these technologies in a more routine way is now possible. While not every practice of bioremediation qualifies as a "technology" according to the program's definition, it does appear worthwhile to evaluate those processes that qualify, including process units that can become part of a more complex treatment train to meet the needs of a particular situation.

The Case for Certification

In contrast to field demonstrations that establish performance under conditions of a single site, certification includes consideration of the range of environmental conditions and media ("matrices") upon which a technology can effectively perform, and generally attempts to predict performance within this "operating envelope." Certification therefore facilitates acceptance of a technology over a range of applications. This in turn should facilitate regulatory permitting.

For these reasons, Cal/EPA convened a multidisciplinary work group of scientists and engineers to develop recommendations to Cal/EPA on criteria and process standards for the certification of bioremediation technologies. The group met from April to July 1996. This Report is the result of this effort. It has been submitted to peer review by outside technical experts. The Work Group has considered their comments. It either accepted the comments or clarified its position, as the case may be, in this updated Report which is now made available to stakeholders for review. After this additional review, a guidance document for applicants will be prepared. At the same time, a reference laboratory is being established which will assist the program with the verification of submitted testing data and make available state-of-the-art diagnostic technology.

As presently written, the Work Group's recommendations address only microbial remediation processes. While phytoremediation processes are not excluded from the program, criteria for these were not considered at this stage, nor were considerations on cost-effectiveness included in the Work Group's terms of reference. The term "certifying body" in this Report refers to Cal/EPA, its departments and boards authorized to issue technology certifications, and other bodies who may evaluate technologies under reciprocity agreements with Cal/EPA.

Purpose of this Document

This document is the result of a pioneering effort to assist Cal/EPA in extending verification/certification to bioremediation technologies. It delineates information needs, minimum criteria to be met, and a process to be followed in demonstrating possible benefits from the use of a technology as the basis for performance certification and regulatory relief. It was not the intent of the Work Group to change the verification/certification processes developed within Cal/EPA but rather to provide technology-specific guidance for implementation. Technology proponents seeking Cal/EPA certification should consult the process documents available from Cal/EPA certifying bodies.

The Work Group recommends that Cal/EPA build a consultative mechanism for addressing bioremediation technologies and to avail itself of state-of-the-art diagnostic methodology to verify, as it deems necessary, proponents' claims regarding the scientific basis of a process, to verify claims regarding performance and reliability under specific field conditions, and to make reasonable extrapolation to conditions not covered in actual field trials.

The Work Group finds that it is the proponent's duty to demonstrate the benefits and safety for Society, public health, and the environment from the use of a bioremediation technology. To assist both the proponent and Cal/EPA, the Work Group also enumerates the diagnostic tools which can provide the missing answers if applied in the appropriate combination and on the appropriate experimental scale. The Work Group has refrained from mandating specific tests. Neither did the Work Group specify which information needs are for the proponent to meet and which are met in verification studies undertaken by, or on behalf of, the certifying body. If a technology is based on a previously established analogous or "parent" technology, quality-assured data from previous implementations of these technologies can be considered by the certifying body. Within the framework established by the Work Group, the certifying body should determine specific information needs and how best to meet them.

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REPORT BY WORK GROUP: RECOMMENDATIONS TO Cal/EPA ON THE CERTIFICATION OF BIOREMEDIATION TECHNOLOGIES

1 Performance Standard - Acceptance Criteria

1.1 Only technologies which reduce risks to health and the environment should be certified.

1.2 Upon achieving acceptable reduction of risk, certified technologies should contribute to the conservation or restoration of natural resources.

2 Topics of Evaluation

What follows is the recommended scope of evaluation, or the areas that should be addressed in the evaluation of bioremediation technologies by Cal/EPA or by other certifying bodies for the results to be acceptable to Cal/EPA.

2.1 General

2.1.1 Certification should be for systems that meet the definition of "technology" cited in the introduction to this Report. The overall system and its components, *i.e.*, equipment, materials, necessary operating skills, and quality assurance and monitoring aspects as they apply to manufacture, service, and use of the system should be subject to evaluation.

2.1.2 To be admitted to verification, a technology should be sufficiently developed for full-scale operation and either commercialized or ready for commercialization after verification/certification. Field verification should be on a scale judged to be field-relevant. Depending on the technology and the variables to be tested, field tests can be supplemented by bench-scale experiments.

2.1.3 The proponent should have control or the right to the use of the technology through patents (either secured or applied for), or through agreement, license, or franchise, as appropriate to assure proper attribution of the technology.

2.1.4 Eligible for certification should be (a) comprehensive treatment methods and procedures employing biological organisms, *i.e,* technologies that can stand alone to achieve remediation goals, (b) biological waste treatment that is part of a comprehensive treatment train including "polishing" technologies to improve the end products of another treatment or pretreatment, and (c) technologies which enhance or improve the performance of a bioremediation treatment, practice, or technique. Individual technologies in these categories may be referred to as "comprehensive treatment technology," "auxiliary bioremediation technology," or "bioremediation enhancement technology," respectively.

Nothing in the certification program should detract from the continued application of simple, proven biological techniques approved by the appropriate regulatory bodies. This includes the design and delivery of individual treatment solutions to individually assessed problems by qualified professionals after evaluation of all relevant variables. These designs may or may not include one or several technologies as defined above. Certification should not circumvent the normal permitting process but facilitate it. For a position statement on this issue, see Section 5.2.

2.2 Efficacy

The following criteria should be applied to determine efficacy of a bioremediation technology. *Screening criteria* are those supported by evidence provided by the technology proponent *for acceptance into the verification/certification process* and to develop performance claims. (As stated here, the materials submitted by the proponent for "screening" include the results of in-house validation studies.) The remaining information needs are the subject of *verification* in independently conducted, quality-controlled studies. The use of prior studies and pre-existing data for verification purposes should be judged by the certifying body on the basis of the quality and independence of these studies and data.

2.2.1 Screening criteria should include the following:

2.2.1.1 That biological processes are necessary to achieve the desired risk reduction, or, for an auxiliary bioremediation technology, that it reduces risk by enhancing a biological process;

2.2.1.2 That the process (a) has novel and unique features, or (b) is a significant improvement of, or an improved combination of, known and accepted bioremediation techniques, or (c) that the process, through novel and unique features, enhances or improves a non-biological treatment process through a biological reaction, or enhances a biological mechanism of remediation. Certifying bodies should not assign priority to the verification/certification of technologies that are established and commonly available at this time, except that significant, novel applications of such technologies may be considered;

2.2.1.3 That the mechanism of action (the scientific and engineering principle) of the technology is sufficiently established in textbooks, peer-reviewed journal articles, patents, or technical documents (including data) of sufficient quality to pass review by technical experts;

2.2.1.4 That the proponent has conducted both laboratory-scale and field pilot-scale experimentation demonstrating that the technology operates as claimed (with regard to its scientific basis) against a well-defined contamination target and that remediation was achieved as measured by acceptable methods and in a reproducible manner.

2.2.2 The following criteria will generally be met through technology verification; they should be a condition for certification:

2.2.2.1 Demonstration, through experimentation on a field-relevant scale, of a range of

appropriate operating conditions for defined types of contaminants, interfering substances, and media (matrices), and that metabolites inhibitory to the process either do not form, or their level is controlled through appropriate measures.

2.2.2.2 Delineation or proof, to the extent needed, of the effectiveness of a specific technology, by demonstrating that the microbial communities in the process medium, whether pre-existing, added, or enhanced, are responsible for carrying out the intended process or processes. If performance claims are related to amendments of existing microbiota, the existence and actions of the existing and the added components of the microbiota, singly and combined, should be determined through appropriate control experiments.

2.2.2.3 For comprehensive treatment technologies: Evidence of significant risk reduction through (a) reduced concentration of the target contaminant or contaminants and (b) reduced hazard from end products or persistent byproducts, and no increase in risk from the remaining microbiota as compared to those originally present.

2.2.2.4 Ranges of experimental parameters (such as temperature, nutrients, pH, dissolved oxygen, contaminant sorption) under which the proposed process is expected to operate, and the technical basis for the operation of these parameters.

2.2.2.5 Analysis to demonstrate that the proposed technology will operate for a reasonable amount of time, and that it will process a significant volume or mass.

2.2.2.6 Experimental evidence that relevant material balances and electron (redox) balances have been established within such limits of accuracy as is necessary to support the proponent's performance claims or to support the requirement of significant reduction of risk.

2.2.2.7 Experimental support of qualitative and quantitative claims concerning the degree of mineralization of the target pollutant(s), and/or the conversion of organic and inorganic pollutants to non-toxic, non-mobile forms, as applicable.

2.2.2.8 Acceptable methods for disposal and/or monitoring of the residues produced, and acceptable methods for monitoring the site after treatment is complete, especially for technologies that bind pollutants into an immobile or slow-release phase.

2.2.3 To the extent that performance data are available from established, sufficiently related ("analogous" or "parent") technologies that can support performance claims of the proposed technology, these data can be submitted by the proponent and considered by the certifying body in support of these claims. (See Section 5.1 for information required.)

2.2.4 All analytical methodology that is used to support the proponent's performance claims should be technically sound and scientifically defensible, appropriate for the intended purpose, reproducible, and verifiable. Technology proponents should understand that reliance on regulatory methods for the analysis of hazardous waste is not usually sufficient to establish material and electron balances for bioremediation processes (including balances of carbon, nitrogen, sulfur, or chlorine, as may be appropriate). Recent developments on testing methodology are highlighted in Section 5.6. The discussion in Section 5.6 is not exhaustive, but the Work Group believes that techniques

such as these, if readily available and used critically, can greatly increase efficiency in the verification of bioremediation technologies.

2.3 Reliability

Evidence should be required to show that:

2.3.1 Sound engineering principles are applied to control operating conditions where needed;

2.3.2 Methods have been developed and found effective to appropriately monitor the process and its critical components (including biological) sufficient to assure effective and stable operation;

2.3.3 Criteria for minimum acceptable performance can be set in terms of these monitoring techniques for each field implementation of the technology, and plans are available for corrective actions by operating staff in case of process malfunction;

2.3.4 Standard operating procedures are available and put in place for orderly startup and shutdown of the process, handling of materials, and the disposal of residues (see also Sections 5.3 and 5.4);

2.3.5 Backup, auxiliary, or containment systems are available as appropriate to protect groundwater or other resources or receptors.

2.4 Protectiveness

2.4.1 The technology proponent should present evidence to show either that the microorganisms added, enhanced, or enriched in the process are generally regarded as safe (GRAS), or if not GRAS that, under the conditions of use, there is no adverse effect on the public health, operator health, or the environment.

2.4.2 Whenever exposure of humans, protected animals, or plants to recognized pathogens or opportunistic pathogens is a possibility, the technology proponent should be required to demonstrate that sufficient protective measures have been designed into the devices and/or processes used to prevent adverse effects during normal function or failure.

2.4.3 The technology proponent should present evidence to show that additives, metabolites, end products, or stable byproducts that may be formed do not present a hazard, or that adequate protection from these hazards is provided.

2.4.4 The technology proponent should present methodology to prevent safety or environmental hazards from developing during any process malfunction, shutdown, or startup, including the handling of materials and the disposal of all effluents and residues.

2.5 Quality Management

2.5.1 The technology proponent should present in-house quality management plans and testing criteria used in the development, production, and service of the technology. (Plans established for ISO-9000 series certification could meet this requirement.)

2.5.2 The technology proponent should present a quality assurance program to be implemented and adhered to by users of the technology. Quality assurance plans should allow operational flexibility as may be necessary to meet treatment objectives. The method of monitoring and documenting the method of adherence to quality plans should be specified. (This may include oversight by regulatory bodies and/or oversight by, or on behalf of, the certifying body in the case of an amended certification being sought on the basis of new, successful implementations, as described in Section 5.5).

2.5.3 The conditions met by the technology proponents in demonstrating satisfactory performance to obtain certification should also be specified by the proponent for practical uses of the technology for certification to remain in force.

3 Process Guidance

3.1 Expert Teams

Before a bioremediation technology is considered for detailed evaluation, verification, or certification, materials submitted by the proponent should be reviewed by a team of experts to determine (a) areas of concern apparent from the materials provided by the proponent, (b) areas requiring substantial additional evidence, and (c) special areas of inquiry related to the nature of the technology. Proprietary data may be required to be submitted by the technology proponent. If marked accordingly, they should be protected from public disclosure.

3.2 Evaluation Teams

Evaluation teams should consist of qualified professionals supported by specialists as needed. Members and specialists should be drawn from the following fields of study as appropriate for the technology and as determined by the key parameters and process controls relevant to the technology: general and environmental microbiology (including bacteriology, mycology or other specialties as appropriate), public health microbiology, environmental epidemiology, chemistry (environmental, analytical), toxicology, engineering (mechanical, chemical, civil, environmental), earth sciences (geology, hydrology, hydrogeology, geochemistry, soil science), and industrial health. Other specialists should be included as necessary on a case-by-case basis. The team should include a member trained in quality assurance appropriate to the technology. Representatives of regulatory agencies having jurisdiction should be given the opportunity to address permitting aspects as they relate to field demonstrations or legal impediments to full-scale applications of the technology, if any. Selection of review team members should be based on a view of the technology as a system in the broadest terms.

3.3 Certification Outlook

The initial evaluation under Sections 3.1 and 3.2 should result in a clearly formulated mutual understanding with the technology proponent on the overall prospect for certification, additional criteria to be met, time and effort required for verification and certification, and possible roadblocks.

3.4 Field Verification

Each verification should include one or several field trials on a field-relevant scale under a plan approved by the certifying body and under the supervision or observation by the certifying body, or under the supervision of a qualified, independent operator or agency acceptable to the certifying body, and under conditions and uses representative of those to be covered in the technology certification. These trials should consider heterogeneity of the process medium, the activity and fate of any added or indigenous microorganisms, the disappearance of substrate and the appearance of intermediate and end products, the effects of environmental conditions and operating parameters on the efficacy of the microorganisms, and the operator's ability to monitor the efficacy of the biological process. Controls on the same scale as the trials are necessary and should be subject to the same level of evaluation as the test system to allow distinction of the proposed biological mechanism from other possible mechanisms. All deviations from the testing plan, and corrective measures taken, should be documented and justified. The field trials may be supplemented with supporting laboratory-scale and pilot-scale experiments as appropriate. For additional discussion see Section 5.5.

3.5 Quality Plans

As part of developing a field testing plan, *data quality objectives* and a *quality management plan* should be adopted in advance of the actual field trial and followed throughout the trial. All data obtained should be available for review by the certifying body. Determination of successful bioremediation in a field trial should be assessed according to criteria provided in these Recommendations and other appropriate references, such as:

(a) Rittman, B., ed. In Situ Bioremediation: When does it work? Washington, D.C.: National Research Council, 1993.

(b) U.S. Air Force Center of Excellence, Technology Transfer Division, Brooks Air Force Base, TX: Technical Protocol for Implementing Intrinsic Bioremediation with Long-Term Monitoring of Fuel Contamination Dissolved in Groundwater, Rev. 0.

(c) Interstate Technology and Regulatory Cooperation Work Group: General Protocol for Demonstration of In-Situ Bioremediation Technologies, c/o Office of Pollution Prevention and Technology Development, California Department of Toxic Substances Control, P.O. Box 608, Sacramento, CA 95812-0806;

(d) American Society for Testing Materials (ASTM): PS 3-95; Provisional Standard Guide for Accelerated Site Characterization for Confirmed or Suspended Petroleum Releases.

3.6 Evaluation Reports

The responsible party should be determined in advance of the trial. Draft evaluation reports

should be reviewed for adequacy and completeness by the team of experts in the fields enumerated above, before the certifying body makes a decision.

3.7 Data Quality Requirements

All data that are critical to the evaluation should conform to quality standards adopted by the certifying body for the purpose. These quality requirements should also apply to third-party data and all third-party studies conducted prior to the evaluation of the technology under the certification program.

3.8 Approval for Genetically Engineered Microorganisms

For technologies which employ genetically engineered microorganisms, the certifying body should require approval for using the microorganism(s), including the intended use, from the U.S. EPA as well as State and local agencies having jurisdiction. Such approval should be obtained before an application for certification is considered and a verification study is undertaken. Restrictions on the use of the organisms should apply as determined by the agencies having jurisdiction over the use of the organism(s). For references, see Section 5.3 of the Appendix.

3.9 Certification Conditions

Consistent with the provisions of California statute (Sec. 25200.1.5, California Health and Safety Code), the scope and conditions of certification should reflect the range of conditions under which successful performance has been demonstrated with data that meet quality standards. In the certification, conditions of safe, reliable, and effective operation should be specified. The certification may specify additional conditions under which reliable performance can be expected based on the results of successful treatability and pilot studies; certification may also prescribe that such studies precede every use of the technology. Amended certifications should be issued as successful performance in the field is demonstrated under different conditions through data that meet the program's quality standards. Certifying bodies should place an expiration date on each certification and establish criteria to restrict or revoke a certification in case of significant failure of a certified technology, unless such a failure can be attributed to failure by the operator to follow proper operating procedures or adhere to the restrictions or quality requirements of the original certification.

4 Testing Capability

The certifying body should have available a capability for authoritative, independent testing in the field of bioremediation (reference laboratory). If this function is carried out by contract organizations, these should be free from conflict of interest and operate under a quality system acceptable to the certifying body. All testing procedures should be documented and documentation kept current at all times.

4.1 Scope of Testing

Testing capabilities should, as a minimum, include the characterization of chemical inputs, outputs, intermediate and byproducts, characterizations of structure, function and dynamics of the relevant microbial community, and bench-scale simulations to confirm, as the certifying body deems necessary, the biologic and process system principle(s) on which the technology is based and critical operating parameters ascertainable on this scale of experimentation.

4.2 Principles of Testing

4.2.1 Reference testing under the direction of the certifying body should use methods that meet quality standards and that are endorsed by regulatory bodies or professional organizations, or are based on publications in the peer-reviewed literature.

4.2.2 Apart from the use of established methods, preferred methods for the characterization of microbial community structure, function, and dynamics are those that allow qualitative and quantitative measurements either in real time or close to it. The testing should be designed to verify performance under realistic conditions in the most efficient and reliable manner. In addition, preference is given to methodology that avoids disturbing the microbial community except when it is intended to study the potential for microbial selection and enrichment. Careful attention should be given to the representative sampling of heterogeneous systems.

4.3 Specific Measurement Techniques

4.3.1 Microbiological Methods may include those described in Section 5.6 as well as other state-of-the-art versions of established methologies of measuring biomass, metabolism, gas exchange, microbial speciation, and microbial biochemical capabilities, as appropriate and as necessary to verify the proponent's performance claims and other essential attributes of the technology. It is the purpose of the proposed reference facility to provide testing to the extent that it is necessary and not readily available, and to act in a reference (referee) capacity. A detailed listing of methodologies established at the reference facility should be maintained together with operating procedures and quality assurance protocols.

4.3.2 Chemical Methods should include, but should not be limited to, regulatory methods (e.g. U.S. EPA SW-846 for solid and hazardous wastes, other U.S. EPA-approved methods, APHA Standard Methods for the Analysis of Water and Wastewater), methods for soil characterizations (TOC, surface, adsorptive properties) and methods for the characterization and measurement of chemical intermediates and dead-end metabolites. All methods should meet the requirements of Section 4.2.1 (See also ANSI/ASQC E4-1995 Standard for Environmental Data Collection and Environmental Technology Programs. American Society for Quality Control.)

5 Appendix

The following position statements were prepared by Work Group members and were accepted and made part of this Report:

5.1 Existing Bioremediation Techniques, Parent Technologies, and Prior Implementations

The outline below represents a compilation of descriptive elements and operating variables for bioremediation systems. Well-packaged "parent" technologies with reliable performance data should not be excluded from certification; in fact, such performance data from such a technology could buttress the case for certifying a technology derived from it.

In the following we list a minimum of data elements necessary to report on implementations and performance of bioremediation technologies. Quality-assured information including these data elements should be required by the certifying body for these to be considered "parent" technologies or to document a prior successful implementation of an applicant technology. The listing below is dynamic and does not claim to be complete.

(a) Technology type, technology name and description, identification of operator, place and time of implementation or trial, contact person for project. Identification of contractors; contract laboratories and their certification status.

(b) Jurisdiction or independent agency overseeing the implementation or trial.

(c) Medium treated, type and description of reactor.

(d) Narrative description and schematic of equipment and process train, with site map.

(e) Volume treated and scale of project (pilot-scale, full-scale).

(f) Names of contaminants including chemical form; concentrations and distribution of contaminants, and analytical methods used.

(g) *In-situ* processes: site geology and contaminant depth. Soil characteristics measured (soil type, sieve analysis, total organic carbon content, bulk density, moisture content, natural microbial community)

(h) Aquifers and groundwater: hydraulic conductivity, porosity, flow direction and velocity, microbial community, total dissolved solids, pH, dissolved oxygen, oxidation-reduction potential, organic matter.

(i) Nutrient and electron acceptors present (excluding amendments); analytical results.

(j) Microbial activity present; how proven? Which control experiments were carried out?

(k) Aerobic, anaerobic process? Which electron acceptors were involved?

(l) Amendments applied and amounts used (microorganisms, nutrients, electron acceptors).

(m) Microbiological and chemical parameters monitored, and results obtained.

(n) Contaminant reduction and risk reduction obtained. After-treatment values of contaminants, nutrients, electron acceptors, pH, redox status.

(o) Disposition of residuals.

(p) Health-related measures: personnel, pathogens, and chems monitored and results obtained.

(q) Project cost.

Analytical results should be presented certified as to their completeness, with indication of the analytical method, and with QA/QC results. The certifying body may specify a preferred format in which to present this information.

5.2 Cal/EPA should not discourage the Use of Professional Services and Applications of Simple and Low-Tech Biological Processes for Remediating Sites

Biological processes have long been recognized as potentially the most cost-effective solutions for treating many types of wastes and waste sites. A tremendous volume of data is generated annually concerning biological treatment of chemical wastes, perhaps even as much as for all other non-biological treatments combined. Much of the interest of the public and engineering consultants concerning bioremediation is directed towards research that has been sponsored by state and federal agencies and supported by government funding.

California leads the nation in the evaluation of environmental technologies through many activities, including the environmental technology certification programs that are run by organizations within Cal/EPA. These certification programs are designed to evaluate and verify the performance of technologies that treat, reduce, or eliminate wastes. Biological treatment technologies, including those for site remediation, are eligible for certification under California's existing programs.

A biological “technology” involves the utilization of a system, and/or additions of specialized biological agents and/or amendments capable of reliably accomplishing the desired result. For example, the making of wine and brewing of beer under controlled conditions, are techniques that create physical conditions that allow common microorganisms to function as planned. Changing either the raw materials or the physical conditions surrounding the fermentation process has a consistent and predictable effect on the performance of the microorganisms and on the product. Many pharmaceutical products are created through technologies that allow microorganisms to generate beneficial chemicals as end products. The treatment of industrial and municipal wastes can be accomplished by engineering technologies that allow and sustain the performance of biological communities capable of degrading wastes. These are but three examples of proven techniques that utilize biological organisms to accomplish a desired goal.

These biological processes might not be considered technologies in a strict enough sense to allow certification under California's environmental technology programs. One example would be the natural or intrinsic bioremediation (“natural attenuation”) of chemicals as a site cleanup practice. While monitoring technologies are deployed to

collect and analyze data characterizing and quantifying the bioremediation process, much of the work would actually fall into the category of providing a professional service rather than application of a technology. In general, provision of professional service in the area of bioremediation is characterized by application of commonly available techniques, materials, measurements, or experience to cause, enhance or document biological processes that treat wastes. Professional training and knowledge are required to allow a technology to be applied under a certain set of conditions. The rendering of a professional service, however, is not to be confused with the technology itself.

As Cal/EPA proceeds to evaluate and verify the performance of technologies through certification programs, the agency should not lose sight of the significant advantages that simple, biological processes - typically investigated, quantified and relied upon as a result of professional services - can offer the environmental industry. Cal/EPA should continue to evaluate such biological processes through technical programs located in its component organizations, by providing encouragement and support for research and development of promising biological processes, and by facilitating the deployment of such processes and associated state-of-the-art monitoring technologies where they are shown to be adequate and cost-effective when compared with other approaches.

Nature has developed many adaptive mechanisms that allow microorganisms to degrade objectionable chemicals in virtually every environment. Where these mechanisms can be encouraged or harnessed by simple, low-tech operations to produce reliable results, they should be considered along with certified technologies. But we should emphasize that intrinsic bioremediation and other simple processes are cost-effective only if there is sufficient assessment and monitoring technology power to quantify the target contaminant decay rate and the causal contribution of the resident microbial community to contaminant removal or detoxification. Better bioremediation monitoring tools would further public acceptance and comfort with waste treatment processes based on intrinsic bioremediation.

5.3 Public Health Issues Associated with Microorganisms Used for Bioremediation

Using microbial agents to mitigate hazardous chemicals either in the open environment or in an industrial or in a home setting raises public health and environmental issues that must be adequately addressed and resolved. Although most microorganisms do not cause adverse human or animal health consequences, many do. Such consequences may range from minimal to life-threatening; and may result in minimal to potentially catastrophic economic impact.

The range of possible public health consequences includes disease and pathological responses due to exposures to infectious or opportunistic microorganisms, and microbial toxins and allergens. In considering these health consequences, it is necessary to know as much information as possible regarding the history of safe use of the microbial agents, the identities of the microorganisms used, their concentrations, and any information regarding their individual health effects, to the degree known.

If defined, pure cultures of well-characterized microorganisms are used, obtaining this

information may be relatively simple. But crude mixtures of microorganisms obtained from natural sources, such as animal wastes, pond sediments, sewage sludge, or sewage digester tanks, *etc.* are difficult when trying to obtain relevant-information; *i.e.*, it may not be possible to know the identities of all microorganisms in the mixture. Crude mixtures of microorganisms obtained from animal sources, unless passed several times through laboratory culture media (not including tissue cultures), may contain viruses that may not be recognized.

The manner in which these agents are used also determines the scope of possible public health issues that must be addressed. If used openly, is it used as a sprayed aerosol, injected into soil, painted onto surfaces, *etc.*, and are there persistent microorganisms or residuals that may later cause health effects to persons or animals and plants that may come into accidental or casual contact with the microorganisms or their residuals? Will the agent be used in inhabited or recreational-use areas, or is it likely to contaminate the food supply, *etc.*? If used in a contained apparatus, would persons or animals normally come into contact with the microorganisms during routine use, or only on failure of the apparatus? Are microorganisms found in the bioremediated product as a consequence of normal use, or are they found there only on failure of the apparatus?

Microorganisms incorporated into systems for bioremediation may include bacteria, fungi, free-living or parasitic protozoa, and viruses. Before the certifying body can appropriately judge the safety of a bioremediation method or apparatus, the proponent (applicant) must provide sufficient scientific information, including but not limited to the following: (i) the identities and sources of all microorganisms included in the method to the degree known; (ii) results of literature reviews that, in the judgement of the certifying body and agency having jurisdiction, identify a history of safe use, if it exists, for the same material, in a sufficiently similar application; (iii) the presence or absence of health consequences associated with these microorganisms, including factors such as dose, exposure, *etc.* needed to define the scope of health risk; and (iv) description of the methods used to exclude and to verify the absence of recognized disease-causing or opportunistic microorganisms.

Where adequate scientific literature, or a long-standing history of safe use in a similar application as determined by the agency is not available, the proponent must also provide the results of independent studies, judged by the agency to be scientifically sound, to show that adverse public health consequences are either absent or essentially zero to convince the agency of the lack of adverse health consequences of the application.

To assure the credibility and reliability of the scientific information provided, the proponent must also demonstrate to the satisfaction of the certifying body or agency having jurisdiction that the persons responsible for the design and conduct of any scientific studies or for collecting and presenting reviews of scientific literature on health consequences are qualified by education, training, and experience in medical and public health microbiology, environmental microbiology, infectious disease medicine, and medical epidemiology; and that they have published peer-reviewed articles in the field(s) relevant to assessing the health consequences of the proposed application.

It is also recommended that the agency have available to it a well balanced, independent, expert panel of well-qualified doctoral level and other experienced microbiologists and epidemiologists, similarly credentialed, who can determine the adequacy of information provided, and, where necessary, suggest additional information that must be provided in order to determine the relative safety of the application.

Where other agencies may have an interest in, or also may have permitting authority over the proposed application, the agency should also set up a formal consultative mechanism, such as a joint review panel, *etc.* to assure that public health effects are adequately considered together with any and all modifications required by the agencies involved.

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5.4 Toxicology Considerations

The technology proponent is advised to submit a Risk Assessment Statement, preferably prepared by a compentent professional risk assessment team. Such a statement should include the analysis of (1) possible metabolites formed from the target chemical and their potential toxicological significance, (2) potential for altering the environmental fate and transport of some toxic chemicals, metals and their conversion products, (3) potential for increasing the bioavailability of toxics present, (4) expected residual toxic contaminant levels and associated risk after completing the project, (5) potential problems associated with local environmental conditions (*e.g.* leaching to nearby aquatic ecosystems and its effects on sensitive fauna or flora, *etc.*).

In preparing such a statement, past patterns of toxicology problems should be considered,

such as: evaporative loss of small molecular weight halogenated alkanes; the formation of toxicologically potent metabolites; mobilization of heavy metal ions such as mercury at low pH; ground water contamination; selective accumulation of toxic contaminants (dioxins); accumulation of soil-bound residue; toxicological properties of certain soil amendments; and toxicity of co-metabolic carbon sources (*e.g.*, added toluene), noxious odor, organoleptic effects, or any other deleterious effects on humans and ecosystems.

Where adequate scientific literature, or a long-standing history of safe use in a sufficiently similar application as determined by Cal/EPA is not available, the proponent should also provide the results of independent toxicological and fate and transport studies, judged by Cal/EPA to be scientifically sound, to show that adverse public health consequences are either absent or essentially zero.

To assure the credibility and reliability of the scientific information provided, the proponent must also demonstrate to the satisfaction of Cal/EPA that the persons responsible for the design and conduct of any scientific studies or for collecting and presenting reviews of scientific literature on health consequences are qualified by education, training, and experience in environmental toxicology, public health, ecological risk assessment, fate and transport, and environmental chemistry, and that they have published peer-reviewed articles in the field relevant to assessing the health consequences of the proposed application.

It is also recommended that Cal/EPA should supplement the panel proposed in Section 3.2 of this Report as necessary with independent, similarly trained scientists that are well qualified at the doctoral level who can determine the adequacy of the information provided, and, where necessary, can suggest additional information that must be provided in order to determine the relative safety of the application.

Where other agencies may have an interest in, or also may have permitting authority over the proposed application, Cal/EPA should also set up a formal consultative mechanism, such as a joint review panel, etc., to assure that public health effects are adequately considered together with any and all modifications required by the agencies involved.

5.5 What Constitutes Adequate Demonstration of a Bioremediation Technology?

5.5.1 Introduction

Through the Certification Program, Cal/EPA will be asked to evaluate bioremediation technologies not only for the likely performance on a specific site but for the expected performance under a range of conditions. The certification program places the burden of proof on the proponent to provide the data necessary to substantiate performance claims. Cal/EPA should provide, however, to the extent possible, clear and direct guidance as to what constitutes an adequate data set. Experience has shown that this is not an easy task, even for the simplest site cleanup technologies that incorporate well-controlled physical, chemical and thermal processes and rely on conventional, well-proven equipment.

5.5.2 Discussion

To determine that a technology will likely perform as claimed on a given site requires

several types of data and information. These include:

An adequate knowledge about the properties of the media to be treated;

An adequate knowledge about the chemicals to be removed or destroyed and their distributions within those matrices;

An adequate knowledge as to how important process parameters will vary, or will be controlled, to assure or determine performance;

For *in-situ* technologies, an adequate knowledge of the structure of the subsurface as it effects or controls the processes affecting treatment;

An adequate knowledge base about the biological parameters (indigenous) that may compete with the proposed bioremediation technology;

An understanding of how available data collected or generated in small-scale treatability studies allow scale-up to accomplish full-scale operation.

Where a treatment process is deployed that alters or destroys the chemicals of concern, the issue of incomplete treatment arises. Where the treatment process relies on biodegradation, and particularly *in-situ* biodegradation, the degree of complexity is greater. The temporal and spatial variabilities inherent in the biological communities being relied upon to effect treatment require that the performance of a technology be followed somewhat differently than for more conventional cleanup processes. If the microorganisms function to degrade the compounds of concern at all, the potential byproducts and endproducts they produce may require analysis by uncommon methods at no small expense. In short, assuring that microbial agents will occur in sufficient numbers in the exact location of contamination, and be acclimated and capable of metabolizing the chemicals of concern is no easy task.

Conventional technologies often have the advantage of a sufficient amount of empirical data from historical applications to predict performance with little additional study or deliberation. One such example in the site remediation field is that of thermal technologies that, once they achieve a predetermined temperature, will reliably treat contaminated soil as long as the equipment is maintained and operated as designed. The sheer magnitude of the energy inputs for these thermal technologies allows a robustness and a reliability that cannot be matched by any other process or technology.

Inherent in certification of any technology is the concept of risk or possibility of failure. Certification of technologies that are highly dependent on several site or process variables cannot offer the same level of assurance as technologies that are not highly dependent on uncontrollable variables. Where a site remediation technology is being evaluated, there is no standard or minimum criterion that has been developed for identifying an adequate level of demonstration to correlate site characterization results, treatment process parameters, and the probability of successful treatment.

5.5.3 Evaluation Approaches

In light of the difficulties that are associated with bioremediation of waste sites, the following approaches and suggestions are offered to Cal/EPA certification programs.

5.5.3.1 Data Base Approach

One approach to certification is to certify past performances of a bioremediation technology for the conditions and contaminant matrix at these sites. The scope of the certification would be expanded when subsequent field applications show successful performance under different local conditions. One possible consequence of this approach is that the generation of quality-assured data sets would become a part of subsequent applications of the technology; these data sets would be generated for review by the certifying body (see Section 5.1). Until a significant amount of experience is obtained with bioremediation technologies, this situation should be anticipated.

5.5.3.2 Different Site Conditions Versus Different Regulatory Requirements

There are two changing conditions that can plague technology developers in their quest for regulatory acceptance: one is the variability among sites and one is the variability in preferences and practices of regulatory agencies that oversee site cleanup. While a consistent level of treatment might be provided by a particular treatment process, that level may not be considered adequate by all of the various entities overseeing site cleanups. The differences may be site-related (population exposure and/or burden on local ecosystems) or may reflect a regulatory philosophy, or both.

Cal/EPA certification programs should recognize the difference between these distinct challenges to technology development and acceptance. Programs underway to encourage clear documentation of regulatory requirements from California agencies and from other states should be encouraged. In particular, the efforts of the Interstate Technology and Regulatory Cooperation (ITRC) Working Group and related projects should continue to be supported by Cal/EPA.

5.5.3.3 Evolutionary Nature of Technology

The natural course of events in the history of a technology is that its use and utility increase over time, and often this increase is slow. It is unlikely that any technology will remain stagnant in its deployment. Even conventional technologies are the subjects of ongoing research and development to increase efficiency and find new applications; Cal/EPA should continue to respond to these developments by amending certifications as appropriate.

The site remediation industry is about five decades old. The bioremediation industry, as a component of the site remediation industry, is less than a decade old. Cal/EPA certification programs should recognize, in their certifications, that the bioremediation industry is in an early stage of its development. In light of this, Cal/EPA certifications of bioremediation technologies should be as thorough as current knowledge and practice allow, but be conservative in judging the transferability of any technology.

5.5.3.4 Relationship with Fuel Tank Fund

A desirable outcome of the Cal/EPA certification programs is that certified technologies become deployed far more frequently than at present. This situation would benefit the environment, as well as the remediation industry and the certification program. One option

for encouraging the certification of a technology is to offer a "preferred status" at cleanup sites. This status could allow expedited approval and deployment of certified technologies.

Where state funds are to be spent on the cleanup, data for certification might be obtained through cleanups with technologies that are under evaluation by the certification program. These opportunities are most apparent in California's Fuel Tank Cleanup Fund. A preference for using certified technologies for fund-lead cleanups would provide the market-pull types of incentives many have suggested are necessary to allow growth and maturity of the remediation industry.

5.5.3.5 Evaluation of Limiting Factors

Unlike physical/chemical processes, biological processes can be limited by a number of factors that are difficult to control. These may include limitations in delivery systems to provide nutrients or energy to microbial agents, rate-limiting steps in a biochemical pathway, limitations imposed by environmental conditions associated with a site such as soil type, soil chemistry, chemical inhibitions, soil heterogeneity or stratigraphic patterns.

An approach to evaluating the performance of bioremediation technologies might be to identify the limits or boundaries beyond which a successful application might not be reliably expected. For an *in-situ* technology the "limits" might include soil permeability or soil heterogeneity that would preclude reliable distribution of injected nutrients or energy supplies. For an *ex-situ* technology the limits might include the need to thoroughly homogenize soil to a uniform size and size distribution.

This approach provides an objective for technology developers to focus on in their development efforts. This approach also puts a burden on the user of a technology to know that the application in mind will actually fall within the known operating envelope for the technology. Unfortunately, at this time few bioremediation technologies have been tested to the limits of "failure" or "diminished performance." In addition, many technologies have not been deployed enough so that the limiting factors are clearly understood.

The cost of repeatedly testing a bioremediation process at bench or pilot scale can be infinitely smaller than the cost of a single full-scale demonstration. In spite of the potential market for successful technologies there is a clear cost of demonstration that precludes repeated full-scale testing of even the most promising bioremediation technologies. Where the technology is deployed at a cleanup site where a private party is funding the effort, the primary objective will invariably be cost-effective cleanup, not demonstration. At most cleanup sites there is little if any incentive for taking on a demonstration in light of the level of effort, costs, potential impact on compliance schedules and, perhaps most importantly, the risk of failure.

5.5.4 Liability Issues

The demonstration or deployment of a bioremediation technology is no different than any other in that certain liabilities drive - and impede - the project. Liabilities associated with sites come from federal and state laws and programs. In the same way that liability drives

cleanup, it also drives responsible parties to select conventional cleanup technologies.

Some of the "conventional" approaches to cleanup (pump-and-treat, soil vapor extraction) did not receive even a small fraction of the research and development that bioremediation has received, yet these "conventional" technologies moved rapidly into full-scale deployment. This situation is due to any number of reasons. However, one significant reason is that these technologies remove contaminants from the subsurface and are viewed as "safe" when compared with other *in-situ* remedies which require addition of solutions and amendments to the subsurface.

While many liabilities originate with the federal government, Cal/EPA should be aware of the double-edged sword of liabilities at the State level that act to impede testing of new technologies. Opportunities to advance the demonstration of new bioremediation technologies and techniques without compromising State authorities and programs should be pursued by Cal/EPA.

5.5.5 Summary

Cal/EPA has broken new ground in developing a certification program that can consider bioremediation technologies. However, the certification program will be impeded by a lack of data from demonstrations - and even from commercial applications - without additional relief and assistance in encouraging demonstration projects. With shrinking federal funds for many environmental programs, this predicament could worsen dramatically.

Cal/EPA should encourage its own cleanup programs to support and encourage the testing and demonstration of bioremediation technologies. Cal/EPA should actively seek to lower the risks, liabilities and "fear factor" that impede many cleanup technology demonstrations by identifying and designating suitable test beds for conducting bioremediation demonstrations. Federal facilities and military base closings throughout California offer Cal/EPA several potential candidates to consider for "test bed" status.

5.6 State-of-the Art Diagnostic Methodologies for Reference Laboratory Use

Assurance that a bioremediation technology performs under conditions other than those of a single field-scale demonstration usually will require a more thorough understanding of the biochemical mechanism or mechanisms and an understanding of the microbiota involved and their expected persistence, growth, and functioning under various field conditions. New and powerful diagnostic techniques are now making it possible to provide better answers to these questions in less time. Using these new techniques requires both specialized materials and experience, adherence to strict quality assurance principles, and the careful interpretation of the results in terms that are meaningful to practitioners and understood by them. The techniques in question are not yet in general use in commercial laboratories. Therefore it is the recommendation of the Work Group that Cal/EPA avail itself of these techniques to complement existing techniques for the evaluation of bioremediation technologies and to provide a reference service as is appropriate and necessary.

5.6.1 Signature Lipid Biomarkers (SLB

Signature lipid biomarker (SLB) analysis based on phospholipid ester-linked fatty acid pattern analysis (PFLA) provides insight into the microbial ecology of the subsurface sediment in a sensitive, cost-effective, and timely manner. Each microorganism has a distinct cell wall pattern and other characteristics, some indicating metabolic state or cellular storage materials.

The hypothesis behind SLB is that there are dynamic changes in the structure and activity of the indigenous microbial community which will be reflected upon exposure to, and degradation of, the introduced contaminants during intrinsic and accelerated bioremediation. One could use changes in the microbial community ecology to assess pollution impact on the population and how they respond to the pollutant. The viable biomass, community composition, and nutritional status of the extant *in-situ* subsurface microbial community can be quantitatively monitored without the necessity for isolation and culture through application of signature lipid biomarker techniques *(1,2)*. These techniques are often used in conjunction with analysis of nucleic acids based on 16S-rRNA sequences which provide specificity in the assessment of community composition and for the potential of specific functional enzyme levels. Together, these analyses can provide a quantitative measure of the total viable and lysed microbial biomass, community composition and nutritional/physiological status of extant microbiota *in situ* without using disruptive techniques.

Recently SLB extraction has been shown to yield purified DNA suitable for enzymatic amplification from the lipid-extracted residue *(3)*. In a recent application, the SLB analysis of subsurface sediments showed a reproducible and marked shift in viable microbial biomass, community composition, and nutritional status with exposure to creosote waste pollution *(4)*. Results of the study showed that a sparse, starving microbial community exhibiting unbalanced growth comprised largely of Gram-positive aerobic microorganisms occupied the uncontaminated vadose zone whereas the pollutant induced an overgrowth of Gram-negative heterotrophs. Shifts in microbial community composition can be induced and monitored quantitatively in soil columns *(5,10,12)* and in the field following nutrient additions *(6)*.

The SLB analysis has also provided significant insight into the response of the microbial communities to *in-situ* environmental conditions in a variety of sample matrices *(7)*. For example, the analysis of periphyton on unglazed tiles incubated in surface water runoff has been utilized an as effective and quantitative multi-species and/or multi-trophic level community toxicity assessment assay (8). Both the Proctor & Gamble and Unilever corporations have built multimillion-dollar artificial streams and have applied the SLB techniques to the study of periphyton to evaluate impacts of their products to fresh water stream ecosystems.

The utilization of SLB in the analysis of microbial communities has been proposed as a monitoring system for subsurface contamination and its natural attenuation *(9)*. Specific pollutants produce reproducible shifts in microbial community composition *(10, 11)*. These shifts can be correlated with increases in effective rates of bioremediation. For example, inraeased rates of petroleum biodegradation have been correlated with increases in biomarkers indicating (a) aerobic growth, (b) viable biomass, (c) abundance of Gram-

negative heterotrophic bacteria, (d) abundance of actinomycetes, and (e) evidence of toxic exposure *(11)*. Increased rates of trichloroethyene (TCE) biodegradation have been correlated with an increased accumulation of aerobic reducing capacity as indicated by increases in the ratio of poly-b-hydroxyalkanoate (PHA) to PLFA *(12)*. The addition of substrates that increase the fortuitous metabolism of TCE, such as methane and propane, also increase proportions of specific components of the subsurface microbial community *(6, 10)*.

A definitive analysis of the rate of return of a pollution-impacted subsurface community to the characteristics of an uncontaminated community has, however, not been determined nor have the factors that effect the rates of return been defined. This concern is being addressed in studies that link pollution assessment, community dynamics, and microbial ecology.

The value of SLB is that it can measure changes in the microbial population without having to isolate the organisms and perform bacterial counts using dilutions. The data base is expanding. It is logical to establish this suite of tests in as a component of bioremediation reference testing to support technology certification in California. It is recommended to perform SLB in conjunction with selected microbial probes such as 16S rRNA. In addition, the Cal/EPA program should employ the Community-Level Physiological Profiling approach developed jointly by the University of California and laboratories of NASA and the Idaho National Engineering and Environmental Laboratory (INEEL) which measures microbial respiratory activities in response to pollutants using BIOLOG instrumentation *(13)*.

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5.6.2 Isotope Monitoring of Bioremediation

The isotopic compositions of subsurface compounds can often be used as natural indicators of the source of these compounds. This can be particularly helpful for tracking the fate of subsurface contaminants in areas undergoing *in-situ* bioremediation. Petroleum hydrocarbons have low stable carbon isotope ratios ($^{13}C/^{12}C$) relative to most other sources of subsurface carbon. These low stable carbon isotope ratios will be reflected in the carbon isotope ratios of CO_2 produced from the aerobic microbial degradation of hydrocarbons (1,2).

There are, however, several situations that can complicate the interpretation of isotope data. For instance, there are other potential sources of subsurface CO_2 that also have low $^{13}C/^{12}C$ ratios, most notably plants that fix atmospheric carbon using the C3 pathway. In this situation, it is necessary to use some other method of differentiating CO_2 produced by degradation of hydrocarbons from CO_2 coming from the root respiration of C3 plants or from subsurface degradation of C3 plant material. To do this, the radiocarbon (^{14}C) content of the CO_2 can generally be used (3). ^{14}C is produced in the upper atmosphere by

interaction of cosmic rays with nitrogen. Living organisms actively exchanging CO_2 with the atmosphere will have a ^{14}C content equal to that of the atmosphere. When they die, they no longer exchange carbon with the atmosphere and the ^{14}C they contain gradually decays until after 60,000 years it has dropped to unmeasureable levels. As petroleum hydrocarbon products are predominantly produced from fossil, ^{14}C-dead carbon sources and C3 plants are generally only a problem in shallow, recent soils, radiocarbon can be used to differentiate between these two sources.

Another potential drawback of stable isotope measurements is isotopic fractionation. Because of the mass differences between the two isotopes, most chemical, physical, and biological processes will have an effect on the isotopic ratios of the products and reactants. For most aerobic microbial processes, the effects are small. For anaerobic processes, such as methanogenesis, however, fractionation effects are significant (4,5). In fact, in areas where methane is being produced from the reduction of CO_2, the $^{13}C/^{12}C$ ratios of the CO_2 can be shifted to very high values. In this case, the ^{14}C contents of the CO_2 can also be useful (6). The ^{14}C contents of natural materials are extremely low, and as a result, the analytical precision is relatively low compared to the analytical precision of stable carbon isotope measurements and the effects of isotope fractionation can not be seen. Fortunately, the range of values we are concerned with is large, so that these measurements can be useful.

Recommendations for the use of isotope measurements are as follows: (1) Perform a background study of the isotopic compositions of other potential sources of byproduct compounds (e.g., CO_2, CH_4) to be measured. (2) Determine whether the effects of isotopic fractionation are significant. For this purpose, measure the isotopic compositions of by-products resulting from microcosm tests performed under similar conditions to those encountered at the site or, if this is not possible, through a literature search of expected microbial processes occurring at a site. (3) Confirm ambiguous results of stable carbon isotope measurements with measurements of radiocarbon (^{14}C) content.

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5.6.3 Genetic Probe Methods

The following is an overview of a suite of nucleic acid-based tools that can be used for verification and validation of bioremediation technologies.

5.6.3.1 Background - The Problem

The development and implementation of bioremediation methods require an accurate understanding of the distribution and activity of microorganisms within the natural environment. Characterization of the structure and function of natural microbial ecosystems are complicated by the nonisotopic dispersion of communities in a medium of considerable physical, chemical, and organismic complexity. In addition, the determination of bacterial species, abundance, and behavior in natural environments are hindered by several factors.

(1) A major fraction (>99%) of the natural microbial biomass consists of noncultivatable organisms. Standard microbiological methods which rely on viable counting and cultivation are useless for the enumeration and classification of noncultivatable soil microorganisms

(2) Soil and aquatic environments are oligotrophic (0.1 to 1.0 mg of growth substrates/liter) frequently forcing organisms into a fragile non-growing postexponential cell state. Even cultivatable bacteria are difficult to "recover" after they enter a postexponential cell state.

(3) The oligotrophic environment, combined with physical heterogeneity, can result in bacterial concentrations as low as 1 to 10 organisms/gram of sample..

(4) Even in cases where *in-situ* biochemical or physiological measurements are possible, the complexity of natural microbial ecosystems frequently compromises the accuracy of these methods. Physiological conditions used to measure the *in-situ* biomass indirectly, or rates of growth and death of one group of organisms, may be inappropriate to measure the contribution of other groups present in the sample. There is an obvious need for state-of-the-art methods which are capable of detecting, quantitating and classifying natural microorganisms at single cell sensitivities.

5.6.3.2 Introduction

Many of the existing obstacles to characterizing natural microbial ecosystems can be overcome by the application of a powerful set of molecular biological tools. DNA/RNA-based diagnostics have revolutionized the identification and quantitation of microorganisms involved in infectious disease. These methods can have a similar impact on ecosystem analysis. The power of DNA/RNA-based diagnostics is a consequence of two features of nucleic acids: (1) nucleic acids can be rapidly and sensitively measured, and (2) the sequence of nucleotides in a target DNA or RNA molecule is unique so that specific hybridization

analyses can be performed using “labeled” complementary gene probes. These techniques are readily automated and are routinely used in state-of-the-art medical diagnostic laboratories. The basis of this technology is the nucleic acid hybridization reaction. Instead of attempting to detect a whole organism (viable counting or staining) or its products (metabolic or antigenic), hybridization detects the presence or absence of a specific nucleic acid sequence. The identification of a microorganism through DNA/RNA analysis relies on the use of a single-stranded DNA probe containing sequences specific to the target organism. If a microorganism in a sample contains DNA/RNA sequences complementary to the probe, the single-stranded target and probe molecules can hybridize forming a double-stranded molecule. To detect the reaction that has occurred, the probe is labeled with a reporter molecule, either a radioisotope, a chemiluminescent or a fluorescent molecule that can be measured with high sensitivity (*i.e.*, subpicogram levels of target can be detected).

5.6.3.3 Technical Implementation and Applications

DNA/RNA-based diagnostic assays offer several advantages over conventional microbial detection systems. Nucleic acids are robust molecules which are considerably more stable than live cells or their proteins. Samples can be treated in a relatively harsh manner and still preserve the integrity of the nucleic acid target. DNA/RNA probes can be used to detect organisms which cannot be cultivated. Hybridization reactions can be monitored by binary assays (dot blots) which give a positive or negative signal, or by analog assays (C_0t or R_0t reactions) which can quantify the concentration of a target molecule. Analog assays are extremely useful for determining the concentration of a given organism within a sample, and for following the response of organisms to changes in their environment.

Ribosomal RNA-based detection systems afford a number of additional advantages in assessing microbial heterogeneity in natural environments. A single slowly growing bacterial cell contains approximately 10,000 ribosomes (ribosomes are the cellular protein synthetic machinery). The detection of a single cell by ribosomal RNA-based systems will be 10,000 times more sensitive than detection by conventional cell plating or microscopic techniques. Ribosomes are composed of two subunits containing 3 ribosomal RNA (rRNA) molecules. The small (30S) ribosomal subunit contains a single 1500-nucleotide ribosomal RNA molecule (16S rRNA) and 21 proteins. 16S rRNA provides both structural and functional activities necessary for protein synthesis.

Because of the antiquity of the protein synthetic process, 16S rRNA has additional utility for discerning phylogenetic relationships among bacteria. 16S rRNAs are ancient molecules, functionally constant, universally distributed, and moderately well conserved throughout the evolution of bacteria and fungi. 16S rRNA is readily purified from natural sources without the use of cloning procedures. Carl Woese was first to realize that the 16S rRNA sequence could be used as an evolutionary chronometer. The 16S rRNA sequence contains several highly conserved regions which can be used for general detection methods, and other "signature sequence" regions containing sufficient sequence variation to be used for group-specific or species-specific detection methods. The 16S rRNA sequence data base presently contains total or partial sequences from over 1,400

microorganisms. 16S rRNA sequence-based oligonucleotide probes can be chemically synthesized that will specifically detect all major known groups and subgroups of microorganisms.

16S rRNA probes can also be used as primers to obtain complementary DNA copies of regions of 16S rRNA, which can be cloned and sequenced to yield precise phylogenetic information about the identity and abundance of microorganisms in a natural sample. This approach uses oligonucleotides complementary to a universally conserved region flanking a 16S "signature sequence" site to prime DNA synthesis across the region of interest. The complementary DNA (cDNA) reaction is allowed to proceed to generate a double-stranded DNA copy of the target region, which can then be cloned and sequenced by standard methods. cDNA synthesis can be performed on rRNA isolated from a natural population of unknown microorganisms. Each of the 16S rRNA reflects the exact abundance of that sequence (organism) among the total rRNA population contained in the sample. In situations where very low concentrations of microorganisms are anticipated, the sensitivity of these rRNA-based methods can be increased by factors of 10^6 to 10^8 through use of the polymerase chain reaction (PCR), and 16S rRNA-specific DNA can be produced in vitro by PCR from natural samples. The PCR-amplified DNA can also be directly sequenced without the need for an intermediate cloning step. By use of these molecular identification tools a complete description of the organisms and their population size can be obtained even if none of the bacteria can be cultivated.

To understand the structure and function of natural microbial ecosystems requires not only methods which assess the distribution of microorganisms, but in addition methods which measure the activity of these populations. Natural population densities are likely to be below the level of detection required by many standard microbial activity assays. However, the response of the microbial population to environmental manipulation (for example, nutrient or oxygen provision) can be monitored by 16S rRNA-based methods. Once a natural population has been characterized (a base line for organisms and their abundance determined), the response of the population to environmental perturbation can be assessed over a period of time. Organisms which increase in numbers must have experienced increased metabolic activity as a result of the experimental treatment and would be implicated in a biotransformation of the input.

It is also possible to use gene probe methods to monitor the presence and abundance of specific contaminant degradation or detoxification genes (methane monooxygenase, toluene monooxygenase, toluene dioxygenase, peroxidases, metal detoxification, *etc*. - *mmo, tmo, tdo, bph, chrA, cop, cat,* and *cad*. Nucleic acid probes directed at these gene targets can be used to follow the emergence and persistence of microbial communities that are involved in these bioremedial processes. Strain-specific gene probes can be used to assess the occurrence and persistence of specialized bioremedial strains.

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